

Original Article

Serum lemur tyrosine kinase-3: a novel biomarker for screening primary non-small cell lung cancer and predicting cancer progression

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Abstract: Purpose: We aimed to determine the expression level of serum soluble lemur tyrosine kinase-3 (sLMTK3) in human non-small cell lung cancer (NSCLC), and to examine whether the sLMTK3 level could be used as a biomarker to screen primary NSCLC and to predict lung cancer progression. Methods: Serum levels of sLMTK3 in 67 patients with primary NSCLC, 28 patients with lung benign lesion, and 53 healthy volunteers were measured by sandwich ELISA. LMTK3 protein expression in NSCLC tissues and normal lung tissues was also detected by using immunohistochemical staining. Receiver operating characteristic (ROC) curve was selected to evaluate the sensitivity and the specificity of serum sLMTK3 level. Results: The mean concentration of sLMTK3 in NSCLC group was significantly higher than in the lung benign lesion group ($P < 0.001$) and the healthy control group ($P < 0.001$). Higher sLMTK3 level was correlated with age ($P = 0.013$), tumor-node-metastasis (TNM) stage ($P < 0.001$), and lymph node metastasis ($P < 0.001$) of NSCLC. In contrast to the normal lung tissues, increased LMTK3 expression was found in the NSCLC tissues, and was mainly located on the cytoplasm and the nuclei of cancer cells. For separating NSCLC from control group, the corresponding areas under the ROC curve (AUC) were 0.947 for sLMTK3 and 0.804 for CEA. With cutoffs of 10.05 ng/ml for sLMTK3 and 5.0 ng/ml for CEA respectively, the sensitivity and the specificity of sLMTK3 and CEA were, 80.60% and 97.53%, 35.82% and 96.30%, respectively, indicating better diagnostic value of sLMTK3. Conclusions: The sLMTK3 level was significantly increased in human NSCLC, and could be used as a potential and valuable biomarker for screening primary NSCLC and for predicting the progression of patients with this malignancy.

Keywords: Lemur tyrosine kinase-3, non-small cell lung cancer, estrogen receptor, tumor biomarker

Introduction

Lung and bronchus cancers is still one of the most common cancers worldwide, and presents the estimated numbers of new cases and deaths will be more than 2.2 million and 1.5 million respectively in the United States in 2013 [1]. Despite multi-model treatment strategies, including surgery, radiotherapy, chemotherapy, biotherapy, and targeted therapy, are used, the death rate of lung cancer is still the first leading cause of cancer-related death both in the World and in the America [1, 2]. The 5-year survival rate of lung cancer, predominantly non-small-cell lung cancer (NSCLC), remains as low as 15% [3]. Therefore, improve-

ments in diagnostics and treatments are urgently needed.

Numerous studies showed that tumor estrogen receptor (ER) and serum estrogen were closely related to the occurrence, development and prognosis of lung cancer [4, 5]. In addition, it has been demonstrated that there is a functional cross signaling between ER, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 receptor (IGF-1R) pathways, and jointly promote the development of NSCLC, combining an inhibitor of the ER pathway with EGFR tyrosine kinase inhibitor (EGFR-TKI) showed enhanced antitumor effects [6-9], this

Table 1. Association of sLMTK3 expression levels and clinical parameters

Parameter	n	Mean ± SD	t	P value
Gender			0.499	0.620
Male	40	16.41 ± 6.24		
Female	27	15.60 ± 7.07		
Age			2.557	0.013
≤ 60	28	18.40 ± 7.39		
> 60	39	14.42 ± 5.36		
Pathological types			0.636	0.527
Adenocarcinoma	46	16.43 ± 6.93		
Squamous cell	21	15.33 ± 5.68		
Differentiation			0.299	0.766
Well-Moderate	32	15.83 ± 6.56		
Poor	35	16.31 ± 6.61		
TNM stage			4.622	< 0.001
I-II	49	14.13 ± 5.71		
III	18	21.41 ± 5.76		
Tumor size			1.509	0.136
T ≤ 3.0 cm	33	14.87 ± 6.02		
T > 3.0 cm	34	17.26 ± 6.90		
Lymph nodes metastasis			4.302	< 0.001
Positive	35	19.01 ± 6.00		
Negative	32	12.89 ± 5.61		

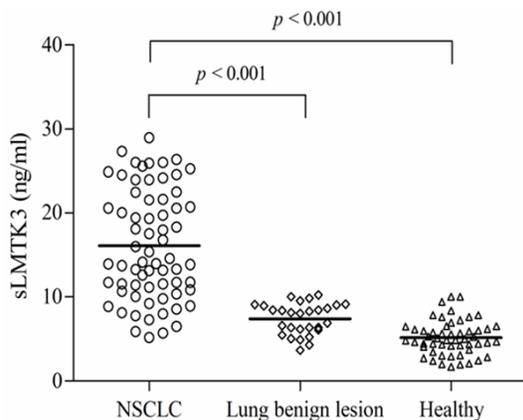


Figure 1. Serum sLMTK3 levels in patients with NSCLC, lung benign lesion and healthy controls. Significantly elevated sLMTK3 levels were found in NSCLC patients compared with that in patients with lung benign lesion or healthy controls ($P < 0.001$).

may possibly providing rationale for combining anti-estrogen therapy with EGFR-TKI for NSCLC treatment.

The phosphorylation of protein kinases and protein phosphatases is a key event in most

nuclear and cytoplasmic processes. The ability to activate and deactivate proteins via phosphorylation or dephosphorylation is important for cell division, cell differentiation, DNA repair and transcription. Tyrosine kinases are particularly important today because of their implications in the treatment of cancer [10, 11]. Lemur tyrosine kinase 3 (LMTK3) belongs to the protein kinase superfamily, tyr protein kinase family. Recently, it was identified as an ER- α regulator associated with endocrine therapy resistance in breast cancer [12]. The abundance of LMTK3 and its polymorphisms were significantly associated with tumor phenotype, disease-free survival (DFS), over survival (OS) and predicted response to endocrine therapies. These findings suggest that LMTK3 expression may be a reliable new biomarker in breast cancer [13, 14]. There were also findings reported that LMTK3 expressed in some other cancers, such as gastric cancer [15] and colon cancers [16], chronic neutrophilic leukemia (CNL) [17]. Meanwhile, our newly reported study showed that the serum LMTK3 could be used as a valuable biomarker for predicting the progression and prognosis of patients with colorectal cancer (CRC) [18]. However, it still remains unknown whether the soluble LMTK3 level in peripheral circulation could be served as an effective biomarker for NSCLC patients.

In this study, we measured the serum levels of sLMTK3 in peripheral blood, and we also characterized the LMTK3 protein in tissues from NSCLC patients, and determined the relationship of the LMTK3 expression with clinical parameters, and to study whether it could be used as a biomarker for screening and predicting the progression of human NSCLC.

Materials and methods

Serum and tissue samples

Preoperative serum samples were obtained from 67 primary NSCLC patients who were pathologically diagnosed and had not undergone any forms of preoperative radiation and/or chemotherapy from June 2011 to April 2013. The clinical and pathological features of

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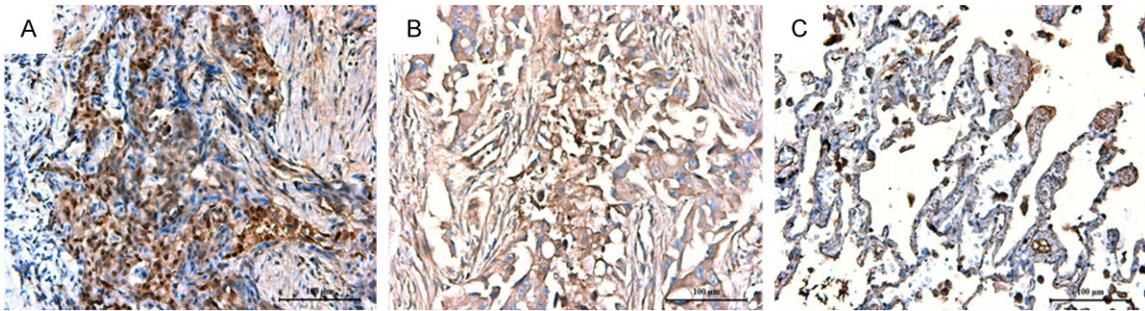


Figure 2. Immunohistochemical analysis of LMTK3 expression in lung cancer and normal lung tissues. A, B. LMTK3 protein was strongly expressed in the cytoplasm and in the nucleus of the lung squamous cell carcinoma as well as the lung adenocarcinoma, respectively; C. LMTK3 protein was weakly expressed in the cytoplasm of normal lung tissues. ($\times 200$ magnification, Scale bar, 100 μm , Lecia DM 2500).

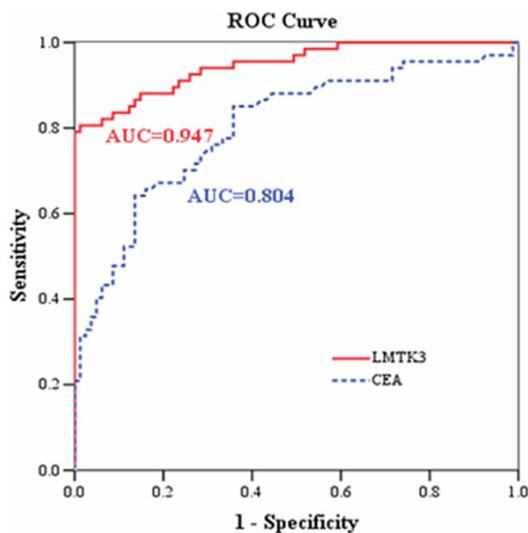


Figure 3. ROC curve analysis of sLMTK3 and CEA as serum markers to separate NSCLC patients from control group.

patients are summarized in **Table 1**. Classification was performed according to the TNM cancer staging system 2007 [19]. As controls, 28 patients with lung benign lesion and 53 healthy volunteers were enrolled. None of the controls had previously been diagnosed with a malignancy. All peripheral blood samples were kept at room temperature for 20 min, and then separated by centrifugation at 4,000 rpm for 5 min. The serum samples were preserved at -80°C before use. Furthermore, some cases of lung cancer tissues and matched adjacent normal noncancerous (control) tissues after surgical resection were fixed by formalin, embedded by paraffin, and then were used in the immunohistochemistry assay. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.

sLMTK3 assessment by sandwich ELISA assay

The serum levels of sLMTK3 was measured by sandwich ELISA using an ELISA kit (Cloud-Clone Corp., Houston, USA). According to the manufacturer's instructions, before measured, reagents and serum samples were remelted at 37°C . The microtiter plate has been pre-coated with an antibody highly sensitive and specific for human sLMTK3. 100 μm of the standards or samples were then added to the appropriate wells. In order to ensure the accuracy of the experiment, each test hole was performed in triplicate. Then, added HRP-labeled avidin and incubated. TMB substract solution was added after thoroughly washed. The color change was measured at a wavelength of 450 nm using a Micro plate reader (Thermo Fisher Scientific Inc., Massachusetts, USA). The concentration of sLMTK3 in the samples was then determined by comparing the O.D. of the samples to the standard cure.

Carcino-embryonic antigen (CEA) assay

CEA, the traditional NSCLC diagnostic marker, was measured by chemiluminescentmicroparticle immunoassay (CMIA) using the Germany Roche Cobas e601 analyzer system and commercially available immunoassay kits (Roche Diagnostics GmbH, Germany).

Immunohistochemical staining for LMTK3 protein

LMTK3 protein expression in tissues was detected by immunohistochemistry staining by using avidin-biotin-peroxidase complex method. All tissue specimens were fixed in 10% neutral formalin, embedded in paraffin and then

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Table 2. Comparison of sensitivity, specificity, and accuracy for sLMTK3 and CEA to distinguish NSCLC patients from control group

Marker	Sensitivity <i>n</i> (%)	Specificity <i>n</i> (%)	Positive predictive value <i>n</i> (%)	Negative predictive value <i>n</i> (%)	Accuracy <i>n</i> (%)
sLMTK3	54/67 (80.60%)	79/81 (97.53%)	54/56 (96.43%)	79/92 (85.87%)	133/148 (89.86%)
CEA	24/67 (35.82%)	78/81 (96.30%)	24/27 (88.89%)	78/121 (64.46%)	102/148 (68.92%)

prepared 5- μ m-thick continuous tissue sections. After the tissue sections were dewaxed, hydrated, antigen retrieval, 3% bovine serum albumin was used to block at 37°C about 30 min. Rabbit anti-human LMTK3 antibody (Cloud-Clone Corp., Houston, USA) was diluted 1:50, incubation overnight at 4°C. Peroxidase-conjugated affinipure goat anti-rabbit IgG (Cloud-Clone Corp., Houston, USA) was diluted 1:100, incubated at room temperature for 30 min, and then coloration by the diaminobenzidine (DAB), stained with hematoxylin, neutral resin mounted at last. Slides were analyzed by two independent pathologists blind to patients' clinical data. LMTK3 immunolocalisation was predominantly found in the cytoplasm and in the nuclei of the cancer cells. Protein expression levels of LMTK3 were determined according to previous reports [14, 15].

Statistical analysis

Statistical analyses were performed by using the GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). The experimental data were show in mean \pm standard deviation (SD). Student's *t* test and One-way ANOVA were used where appropriate. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference. In addition, receiver operating characteristic (ROC) curve was established to discriminate the subjects with or without NSCLC. All the analyses were carried out using the SPSS 13.0 software (SPSS Inc., Chicago, USA).

Results

Abnormal expression of serum sLMTK3 levels in NSCLC

The mean concentration of serum sLMTK3 in 67 NSCLC patients was 16.08 ± 6.54 ng/ml. In contrast, the mean level in 28 patients with lung benign lesion was 7.42 ± 1.87 ng/ml and 53 healthy volunteers was 5.15 ± 2.03 ng/ml. As shown in **Figure 1**, the serum levels of

sLMTK3 were significantly higher in NSCLC group than that in lung benign lesion group or in healthy donors group ($P < 0.001$, One-way ANOVA test).

LMTK3 protein expression in lung cancer tissues and normal lung tissues

We also analyzed the expression of LMTK3 protein in lung cancer and matched adjacent normal tissues by using immunohistochemistry assay, and found that it was significantly increased expression both in lung squamous cell carcinoma and adenocarcinoma, mainly expressed in the nucleus and cytoplasm (**Figure 2A, 2B**). High expression of LMTK3 was detected in 70.00% of NSCLC (7/10) patients, including 71.43% of adenocarcinoma (5/7), 66.67% of squamous cell carcinoma (2/3). While a total of 3 (30.00%, 3/10) normal lung tissues were weakly positive, mainly existed in the cytoplasm (**Figure 2C**).

Correlations between sLMTK3 level and clinical parameters of NSCLC patients

The relationships between sLMTK3 level and clinical parameters of NSCLC patients were demonstrated in **Table 1**. We found that the expression levels of sLMTK3 were significantly correlated with age ($P = 0.013$), TNM classification ($P < 0.001$), and lymph nodes metastasis ($P < 0.001$). However, no statistically significant associations were found between sLMTK3 and clinical parameters of NSCLC patients, such as gender, pathological types, differentiation, and tumor size. In addition, a significantly higher sLMTK3 level was observed in NSCLC patients with tumor size bigger than 3.0 cm to those with smaller than 3.0 cm ($P = 0.136$).

Comparison of sLMTK3 and CEA as tumor markers

We compared the potential for use as tumor markers for NSCLC of serum sLMTK3 with that of CEA. ROC curve analysis illustrated that the

AUC values for sLMTK3 and CEA in patients with NSCLC versus tumor-free controls were 0.947 and 0.804, respectively (**Figure 3**). We decided to designate the cutoff point of sLMTK3 and CEA as 10.05 ng/ml, 5.0 ng/ml, respectively. The sensitivity, specificity, and accuracy of sLMTK3 and CEA to distinguish NSCLC patients from control group were showed in **Table 2**. These data indicate that sLMTK3 is an ideal biomarker for NSCLC.

Discussion

Recent studies have found that LMTK3 was expressed in several types of cancers, including breast cancer [13, 14], gastric cancer [15], CNL [17], and colorectal RC [16, 18]. In our present study, we aimed to investigate the associations between serum sLMTK3 level and patient's clinical parameters of NSCLC patients, and our data support that the functionality of sLMTK3 in lung cancer diagnosis could predict cancer progression, and may be a novel therapeutic target to human NSCLC.

Our study showed that the expression levels of sLMTK3 were significantly increased in NSCLC patients in contrast to the benign group and healthy donors. We further investigated the relationship between sLMTK3 level and clinical parameters of NSCLC patients, and found that the higher sLMTK3 level was significantly correlated with age, TNM stage, and lymph node metastasis, but not with gender, pathological types, differentiation, or tumor size. Shi *et al.* detected the expression of serum sLMTK3 in 60 patients with CRC as well as 53 healthy volunteers by sandwich ELISA and found that the mean concentration of sLMTK3 in CRC patients was significantly higher than that in healthy volunteers, and the sLMTK3 level were significantly correlated with depth of tumor invasion, histological subtype, and TNM stage [18].

In addition, we also compared the expression of LMTK3 protein in NSCLC tissues with normal lung tissues by immunohistochemical staining. Our data demonstrated that high LMTK3 protein expression both in cytoplasmic and nuclear of lung squamous cell carcinoma and adenocarcinoma, and normal lung tissues have three cases weakly expression in cytoplasmic. Moreover, we found that LMTK3 protein highly expressed in tissues were agreed with high expression serum levels of sLMTK3. Previous studies have demonstrated that in primary

breast cancer tissues, high expression of LMTK3 was associated with tumor phenotype, DFS, OS and predicted response to endocrine therapies [13, 14]. Wakatsuki *et al.* also found that LMTK3 polymorphisms rs9989661 and rs8108419 were significantly associated with DFS, OS and time to recurrence (TTR) in gastric cancer, but this may be dependent on the regional differences both in physiology and genetic alterations of gastric cancer [15]. Research also found that in tamoxifen-resistant cell lines, the addition of LMTK3 small interfering RNA (siRNA) increased the inhibitory effect of tamoxifen. LMTK3 siRNA decreased tumor growth of nude mice with human MCF-7 breast cancer tumors [20]. LMTK3 was implicated in endocrine resistance via multiple signaling pathways, for example, by means of reducing autophagy at a transcriptional and translational level, thereby protecting MCF-7 cells, which is an ER α -positive breast cancer cell line, from tamoxifen-induced cell death [21]. MicroRNA-34a suppresses cell proliferation by targeting LMTK3 in human breast cancer MCF-7 cell line [22]. Therefore, LMTK3 is a possible target and marker of breast cancer.

In our study, to evaluate the potential diagnostic value for NSCLC, conventional tumor marker CEA was compared with sLMTK3 in NSCLC patients versus patients with lung benign lesion and healthy controls. ROC curve analysis showed that sLMTK3 is more useful than CEA. Combination assay with sLMTK3 and CEA may be more useful in the improvement of diagnosis of NSCLC. Based on AUC of sLMTK3 and predictive capacity of sLMTK3 for NSCLC occurrence, we propose that sLMTK3 is valuable to differentiate NSCLC patients from tumor-free controls.

In summary, we observed that the higher expression of serum sLMTK3 and LMTK3 protein in patients with NSCLC, and the sLMTK3 level was significantly correlated with clinical parameters including age, TNM stage, and lymph nodes metastasis, and could be used as a non-invasive, blood-based biomarker for NSCLC. However, the underlying molecular mechanisms of LMTK3 involved in NSCLC progression merits further investigation.

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Disclosure of conflict of interest

None.

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