Serum Detection of Thymidine Kinase 1 as a Means of Early Detection of Lung Cancer

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Abstract. *Background: Thymidine kinase 1 (TK1) is a biomarker elevated in several malignancies, including lung cancer. Up-regulation of TK1 is an early event in carcinogenesis and therefore a target for early cancer detection. We have developed a novel Enzyme Linked Immunosorbent Assay (ELISA) to detect TK1 in serum. Materials and Methods: Forty patients with pulmonary nodules and 18 healthy individuals had their serum collected prior to surgery. All samples were analyzed using a radioassay and ELISA. Results: TK1 was significantly elevated in all lung cancer samples. Patients with stage I (n=16) and stage II (n=17) disease had significantly higher TK1 levels than controls. The area under the curve was 0.792, using 4.9 nM TK1 as cut-off, for early-stage lung cancer. The sensitivity and specificity were 75.0 and 83.3, respectively. TK1 concentration was a more sensitive and accurate indicator of lung cancer than TK1 activity. Conclusion: TK1 is significantly elevated in serum from patients with stage I and stage II lung cancer as measured using the established ELISA. This novel TK1 ELISA is both sensitive and specific for the detection of early-stage and advanced lung cancer, and therefore may be an important tool in the management of this disease.*

Lung cancer accounts for the majority of cancer-related deaths in men and women worldwide (1). Currently there are no biomarkers that are truly useful for the early detection or monitoring of lung cancer (2). The only recommended early detection tool for lung cancer is low-dose computed tomographic (CT) scanning; the US National Lung Screening Trial reported 20% reduced mortality for high-risk individuals screened with spiral CT scans (3). Despite its promising potential, there are many disadvantages to this screening method, including cost, radiation exposure and unequal adoption among insurance carriers. The possibility of developing a serum test for early lung cancer detection is attractive due to the ease of obtaining reference samples, as well as the minimal risk involved. There have been many attempts at detecting protein or genetic material in the serum or sputum of patients with lung cancer but these have all failed due to the lack of sensitivity in detecting minute amounts of material in patients with early-stage disease.

TK1 is a well-established cancer biomarker which is elevated in patient serum and tumor tissue of many hematological and solid tumor types, including lung cancer (4-6). TK1 is efficacious as both a diagnostic and prognostic tool since changes in serum TK1 levels reflect a patient's response to treatment and risk for recurrence (7-9). TK1 is elevated in the very early stages of malignancy. In fact, in a health screening of 8,135 individuals, 1.1% of individuals had elevated TK1 levels, in which nearly 90% of those with elevated TK1 had pre-cancerous diseases (10). Included in those non-cancerous individuals with elevated TK1 was one individual who developed liver carcinoma 13 months following the reported high TK1 level. Several other studies have confirmed that TK1 is elevated in pre-malignancy. Guan *et al*. demonstrated that TK1 is significantly elevated in atypical ductal hyperplasia, ductal carcinoma *in situ*, and invasive ductal carcinoma compared to normal breast tissue (11, 12). However, they reported that TK1 was not significantly elevated in usual ductal hyperplasia. This indicates that perhaps elevated TK1 is associated with pre-cancerous diseases which have an increased risk of progression. This appears to be the case and has been confirmed in several large health screenings, including one with 35,365 individuals and one with 11,880 individuals (13, 14). In these health screens, individuals with pre-malignant

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diseases were found in both the elevated-TK1 and normal-TK1 groups, although a significantly higher percentage of individuals with pre-malignant diseases had elevated TK1. The patients with pre-malignant disease with elevated TK1 were more likely to show disease progression within 5 to 72 months compared to those with normal TK1 (8.8% vs. 0.2%) (14). Therefore, non-cancerous individuals with elevated TK1 levels had a 3- to 5-fold increased risk of developing malignancy (14).

Lung cancer is frequently diagnosed in its advanced stages, and TK1 is elevated in lung cancer and other malignancies very early. Therefore, we sought to develop a TK1 Enzyme Linked Immunosorbent Assay (ELISA) to help identify patients with lung cancer much earlier. The successful development of this assay will likely complement the use of CT scans to further identify groups of higher risk patients that may then obtain the necessary imaging.

Materials and Methods

Patient samples. Serum samples from 40 patients with solitary lung nodules thought to be at high risk for either lung cancer or lung metastases were obtained from two tertiary care Hospitals. Serum samples were collected within 2 weeks prior to surgical resection of lung nodules. Serum samples from 18 healthy individuals without known lung disease were also obtained as control samples. Patients enrolled after informed consent under an institutional review board approved protocol. Upon collection, samples were immediately stored as aliquots at −80˚C until analysis.

TK1 ELISA. All serum samples were analyzed using a previously described direct ELISA protocol with slight modifications (15). The antibody against human TK1 used was a highly specific mouse monoclonal antibody (produced by our laboratory) which reacts against an internal fragment of TK1 designated A74. Briefly, 50-μl samples were diluted in carbonate buffer, pH 9.6, and allowed to absorb to 96-well ELISA plates overnight at 4˚C. Wells were then washed four times with phosphate-buffered saline (PBS), pH 7.4, and blocked with 2% bovine serum albumin diluted in T20 PBS Blocking Buffer (Thermo Fisher Scientific) for 1 h at room temperature. Wells were again washed and then incubated for 1 h at room temperature with horseradish peroxidase conjugated antibody to human TK1 diluted in block. Wells were again washed and developed with 1-Step Ultra TMB (Thermo Fisher Scientific) and quenched with sulfuric acid. Samples were analyzed using Synergy HT Microplate Reader (Bio-Tek Winooski, VT, USA) at 450 nm. Samples were run in duplicate and confirmed with at least two independent experiments. A standard curve was constructed using recombinant human TK1 at different concentrations diluted in carbonate buffer. Using the average of the serum samples and the recombinant TK1 standard curve, the TK1 concentration in the serum samples was calculated.

TK1 radioassay. The TK1 radioassay was optimized for human serum samples and run as previously described (16). The reaction mix contained 10 mM Tris-HCl pH 7.6, 2 mM Dithiothreitol (DTT), 5 mM $MgCl_2$, 5 mM NaF, 5 mM ATP, and 5 μM [^{3}H]-deoxythymidine. All serum samples were analyzed using the TK1 radioassay as quadruplets and the data was confirmed with at least two independent experiments. The average of all runs was used for statistical analysis.

TK1 dot blot. Serial dilutions of serum or recombinant TK1 (10 μl) were applied to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and allowed to dry for 1 h at room temperature. The membrane was then blocked with 5% non-fat dry milk (NFDM) diluted in tris-buffered saline (TBS) for 1 h at room temperature. Following three washes with 0.1% Tween T20 in TBS (TBS-T), the membrane was incubated with A74, a highly specific monoclonal antibody to TK1, diluted in block for 1 h at room temperature. The membrane was washed five times with TBS-T and incubated with goat anti-mouse HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for one hour at room temperature. The membrane was washed again and developed using Immun-Star WesternC Chemiluminescence (Bio-Rad).

Immunohistochemistry. TK1 staining of lung cancer and normal tissue was performed as previously described (12). Briefly, tissue slides were deparaffinized, rehydraded and antigenicities were retrieved using 0.01 M sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked, and the slides were washed prior to incubation with 10% normal horse serum. Primary antibody, a highly specific monoclonal antibody to TK1 optimized for immunohistological staining (CB001) was added for three hours at room temperature. Following the primary antibody, slides were washed, incubated with biotin-conjugated secondary antibody, washed and then incubated with streptavidin-peroxidase (ABC kit; Vector Labs Inc Burlingame, CA USA). Slides were then washed, developed using diaminobenzidine (Vector Labs Inc.), and counterstained with hematoxylin.

Statistical analysis. The diagnostic power of this TK1 ELISA was determined by analyzing the area under the receiver operating characteristics (ROC) curve. The best cut-off value was determined by maximizing sensitivity and specificity. The data are presented as the mean±standard deviation (SD) and median±interquartile range (IQR). Student *t*-test and Wilcoxon rank-sum test were used to determine statistical significance, which was set at $p<0.05$. Sensitivity, specificity, positive predictive value, negative predictive value, and odds ratio [±95% confidence interval (CI)] were reported. All analyses were performed using SAS version 9.0 (Cary, NC, USA).

Results

Patients' characteristics. Forty patients who underwent resection for solitary pulmonary nodules suspected as being malignant were enrolled in the study. Thirty-three patients were found to have non-small cell lung cancer with 16 patients classified as having stage I, node-negative (pT1a and pT1b) disease, and 17 classified as having stage II lung cancer. Additionally, seven patients had nodules deemed to be metastases arising from the colon, esophagus, cervix, or prostate. The majority of lung tumors were adenocarcinomas (n=16) with squamous cell (n=7), adenosquamous cell carcinoma (n=2), and atypical carcinoid, bronchioalveolar carcinoma, large cell neuroendocrine, mucoepidermatoid

carcinoma, and sarcomatoid carcinoma comprising the remainder of the specimens. The mean age of patients was 67±12 years. The percentage of never-smokers (n=9) in our data-set was slightly higher compared to the national average of never-smoker patients with lung cancer, at 28% and 10-15%, respectively (17). The patients represent those with operable lung cancer as the tissue banking protocol only included patients who underwent surgical resection.

Specificity of A74 antibody. In order to confirm that A74 can accurately detect native TK1 in tumor tissue as well as serum, we stained squamous cell carcinoma tissues and lung adenocarcinoma for TK1 expression using a highly specific monoclonal antibody (CB001) appropriate for TK1 immunohistochemical detection (Figure 1A, B). TK1 expression was clearly detectable in lung cancer tissue with minimal or no staining in normal lung tissue (Figure 1C). This confirmed several other studies which have reported significantly elevated TK1 expression in lung carcinoma tissue, including pT1 adenocarcinoma tissue, compared to normal lung (4, 6). Furthermore, we determined through dot blot that A74 TK1 antibody detected native recombinant TK1 as well as native cancer-derived TK1 in serum (Figure 1D, E). Native western blot analysis confirmed that A74 detected native serum TK1 at its expected molecular weight, 100-700 kDa (data not shown). This highly specific antibody is unique in its ability to identify unreduced serum TK1. This unique property allowed us to proceed and develop an immunoassay without the need for reducing agents.

TK1 ELISA.

All lung cancer cases: We used recombinant TK1 as the positive control to generate a standard curve for this novel TK1 ELISA. Serum samples from 40 patients with operable stage I and II lung cancer were collected for TK1 analysis. TK1 concentration was analyzed using the TK1 ELISA, while TK1 activity was determined using the traditional TK radioassay, previously optimized for human serum samples (16). The mean±SD of TK1 concentration in operable lung cancer patients and healthy controls was 5.6±1.0 nM and 4.5±0.8 nM, respectively. The median (IQR) for TK1 concentration in serum from patients with lung cancer and healthy individuals was 5.8 (5.0-6.2) nM and 4.4 (3.9-4.6) nM, respectively. There was a highly statistically significant difference between the TK1 concentration in patients and that in healthy controls, as determined by the TK1 ELISA by both the Wilcoxon rank-sum and Student t-test ($p<0.001$). The mean±SD of TK1 activity, as determined by the traditional TK radioassay for lung cancer and healthy controls was 8,866±1029 cpm and 9,575±1579 cpm, respectively. There was no statistically significant difference in TK1 activity between patients and healthy individuals ($p=0.094$). Since our cancer samples represent very early-stage lung cancer, we are not surprised that the TK radioassay could not distinguish major differences in TK1 activity. This indicates that our novel TK1 ELISA is more sensitive and accurate than the traditional TK1 radioassay, especially for early-stage lung cancer.

The best cut-off value for patients with stage I and II lung cancer, as determined by ROC analysis was 4.9 nM. At this cut-off value, there was a highly statistically significant difference between serum TK1 concentration in lung cancer and healthy controls ($p<0.0001$). The area under the curve (AUC) for cancer patients was 0.828 (Figure 2B). The sensitivity and specificity (95% CI) at this cut-off value was 81.8% (64.5-93.0%) and 83.3% (58.6-96.4%), respectively (Figure 2). The positive and negative predictive values were 90.0% (73.5-97.9%) and 71.4% (47.8-88.7%), respectively. The odds ratio (95% CI) at this same cut-off value was 22.5 (4.9-103.2), indicating that patients with a serum TK1 concentration above 4.9 nM had 22-fold increased risk of having lung cancer than those with serum TK1 levels <4.9 nM.

Stage I, node-negative lung cancer: In an effort to determine if our novel TK1 ELISA is able to detect significant differences at the earliest diagnosed stage of lung cancer, stage I, node-negative, we separated the 33 lung cancer sera into stage I and II cases. The mean±SD TK1 concentration in stage I (pT1) lung cancer, stage II lung cancer, and healthy individuals was 5.3±1.0, 5.8±1.0 and 4.5±0.8 nM, respectively; the median (IQR) TK1 concentration was 5.4 (4.7-6.1), 5.8 (5.2-6.4), and 4.4 (3.9-4.6) nM, respectively (Figure 3). According to the Student *t*-test and Wilcoxon rank-sum test, there was a statistically significant difference in TK1 concentration between stage I lung cancer and control samples ($p=0.01$). There was also a significant difference between stage II lung cancer and healthy controls according to both the *t*-test and rank-sum test ($p<0.001$). There was no significant difference by either the *t*-test or rank-sum test between stage I and stage II lung cancer ($p=0.16$ and $p=0.27$, respectively). The mean±SD of TK1 activity in stage I and stage II lung cancer, and healthy controls was 8,816±968, 8,770±1126, and 9,574±7448 cpm, respectively. According to the Student's *t*-test, there was no significant difference between any of the groups. This confirms our previous finding that the novel TK1 ELISA is more sensitive and accurate than the traditional TK1 radioassay.

The best cut-off value as determined by ROC analysis for stage I lung cancer and heathy controls was also 4.9 nM ($p=0.0016$). The AUC was 0.792 (Figure 2A). The sensitivity and specificity (95% CI) were 75.0% (47.6-92.7%) and 83.3% (58.6-96.4%), respectively. The positive and negative predictive values were 80.0% (51.9-95.7%) and 79.0% (54.4-94.0%), respectively. The odds ratio (95% CI) at this cut-off value was 15.0 (2.8-80.4) indicating that patients with TK1 concentrations above 4.9 nM had a 15-fold increased risk of having stage I lung cancer.

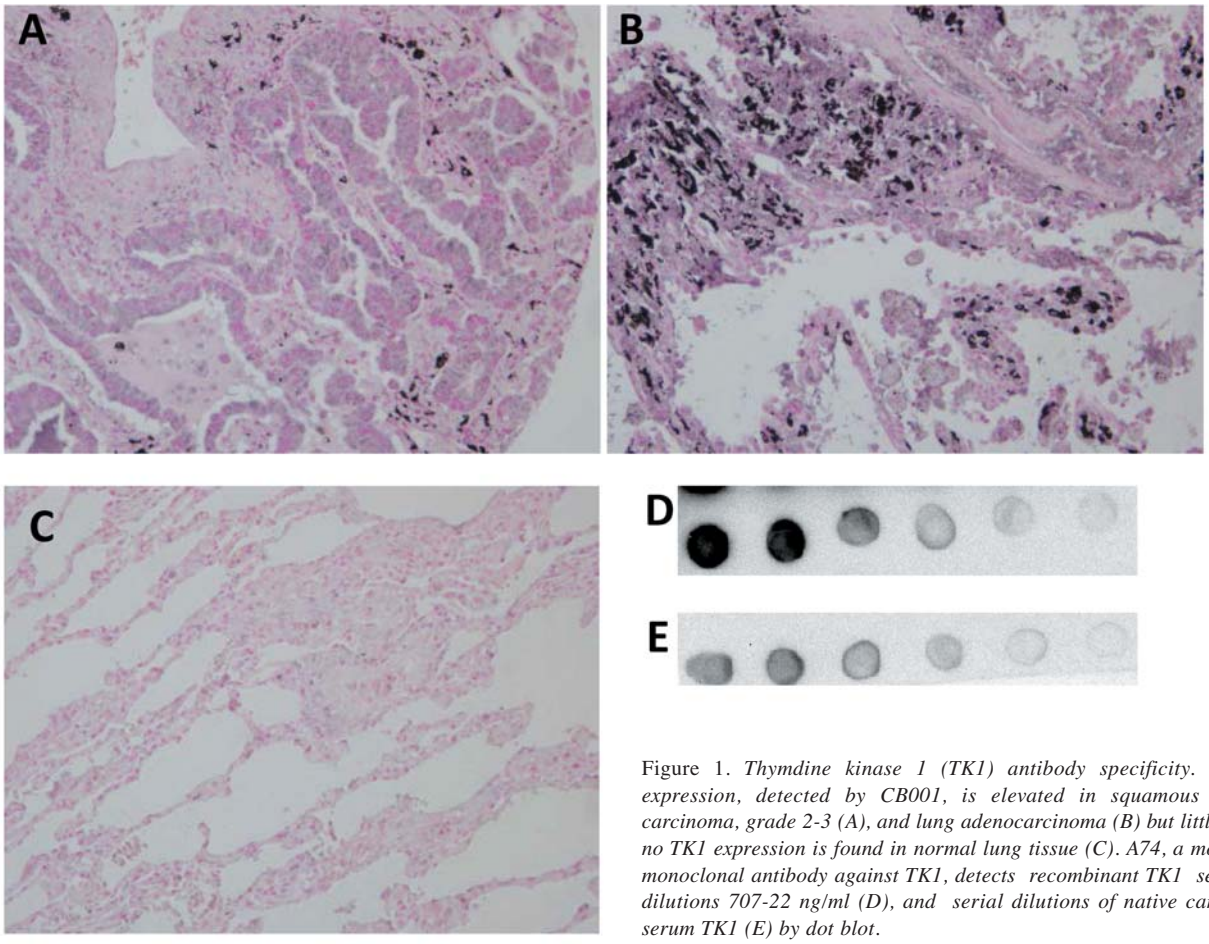

Figure 1. *Thymdine kinase 1 (TK1) antibody specificity. TK1 expression, detected by CB001, is elevated in squamous cell carcinoma, grade 2-3 (A), and lung adenocarcinoma (B) but little or no TK1 expression is found in normal lung tissue (C). A74, a mouse monoclonal antibody against TK1, detects recombinant TK1 serial dilutions 707-22 ng/ml (D), and serial dilutions of native cancer serum TK1 (E) by dot blot.*

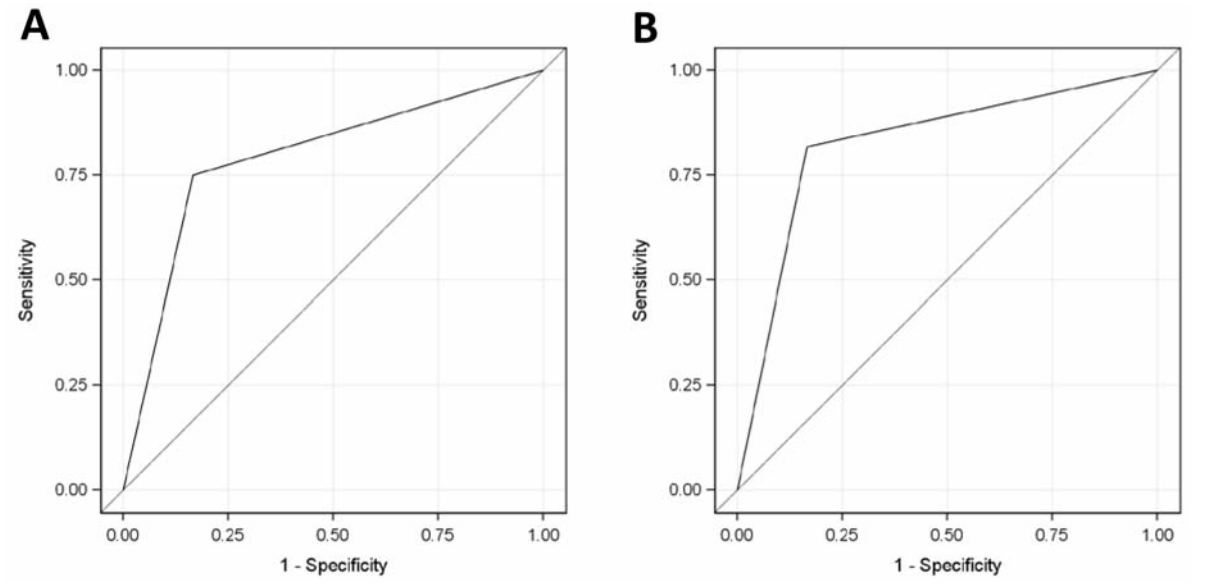

Figure 2. *Receiver operating characteristic (ROC) curves. A: The area under the curve for stage 1, node-negative lung cancer is 0.79, with a sensitivity and specificity of 75.0% and 83.3%, respectively. B: The AUC for all lung cancer cases is 0.82, with a sensitivity and specificity of 81.8% and 83.3%, respectively.*

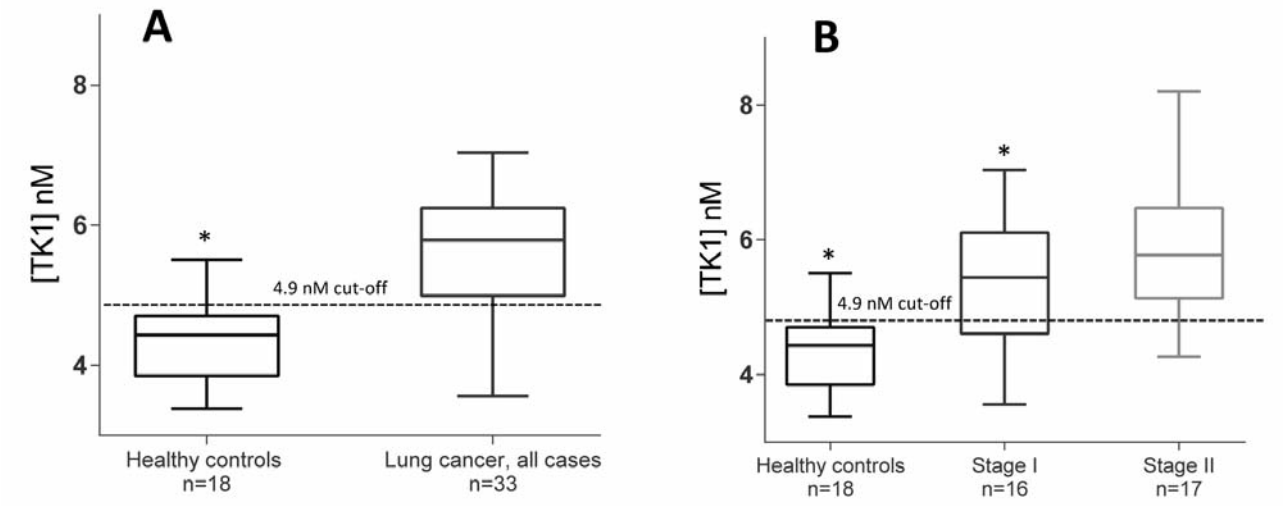

Figure 3. *Box plot of distribution of thymidine kinase 1 in serum in lung cancer cases and healthy controls. The median and interquartile ranges are represented by the center line and box, respectively. The maximum and minimum values, excluding outliers, are represented by the vertical lines. A: There is a highly significant difference in TK1 concentration in serum from patients with operable lung cancer (n=33) compared with normal controls (n=18) (p<0.001). B: There is a significant difference in TK1 concentration in stage I (n=16) and stage II (n=17) lung cancer compared to controls (p=0.007 and p<0.001, respectively). There is no significant difference in TK1 concentration between stage I and stage II lung cancer. *Represents statistical significance.*

Out of the 40 operable lung cancer cases, seven were from patients with lung metastases. According to the Student *t*-test results, these patients had significantly higher TK1 concentrations compared with healthy controls (p=0.0001) but there was no significant difference in TK1 activity (p=0.508) (Figure 4). There was no significant difference in TK1 concentration or TK1 activity between those with primary and those with metastatic lung tumors.

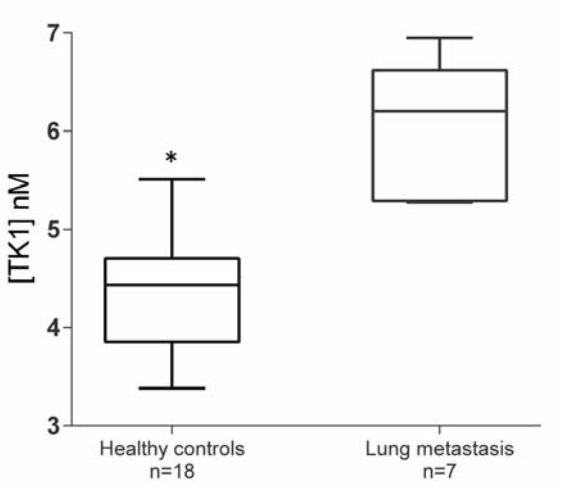

Figure 4. *Box plot of thymidine kinase 1 distribution in serum from patients with lung metastases and healthy controls. There is a highly significant difference in TK1 concentration in serum from patients with lung metastasis (n=7) compared with healthy controls (p<0.001). *Represents statistical significance.*

Discussion

Previous studies have demonstrated that TK1 is a powerful diagnostic and prognostic tool in the serum of patients with cancer. Unfortunately, the clinic has not yet harnessed this potential because of challenges associated with developing a clinically relevant method of detection. A TK1 ELISA, such as the one proposed here, overcomes limitations of using radioisotopes in the traditional TK1 radioassays and problems associated with specificity, sensitivity, and reproducibility. Since the ELISA platform is commonly used in the clinic, a TK1 ELISA may be adapted to other high-throughput immunoassays currently in clinical use.

This study introduces a novel TK1 ELISA which is both sensitive and specific. In addition to detecting significant differences in TK1 between advanced lung cancer and healthy individuals, there was a significant difference in TK1 levels in serum of patients with stage I, node-negative lung cancer and those of healthy individuals, while TK1 activity did not differ. This indicates that our novel TK1 ELISA is more sensitive and accurate than the traditional TK1 radioassay. This confirms other studies which have reported that in lung, breast, gastric, rectal, colorectal, brain cancer, leukemia, lymphoma, and hepatoma, assay of TK1 protein concentration is more sensitive than that of TK1 activity (5).

In ROC analysis, sensitivity of 100% would indicate that even asymptomatic individuals with the earliest stages of lung cancer would have TK1 elevated above the cut-off value. The two ROC curves representing all lung cancer cases and only individuals with stage I, node-negative disease had sensitivities and specificities of 81.8% and 83.3% and 75.0% and 83.3%, respectively. A sensitivity or specificity between 90-99% could not be derived for our data set since very few patients had concentrations above the cut-off values. In subsequent follow-up studies with larger sample sizes and more advanced tumors, it will likely be possible to achieve a sensitivity or specificity >90%. However, the AUC, sensitivity and specificity of the TK1 ELISA is comparable to those for other TK1 immunoassays, which indicates this novel TK1 ELISA may be a reliable method of lung cancer detection (14, 16, 18).

Several studies have indicated that elevated TK1 is associated with risk for disease progression (10, 11, 13, 14, 19). Some tumors have a lower risk of progression and result in a corresponding low TK1 level (20). This is one possible explanation for why some individuals in our study with lung cancer had TK1 levels below the cut-off value. These sub-cut-off values for lung cancer samples lower the overall negative predictive value of the assay. A long-term follow-up study of lung cancer cases with low preoperative TK1 levels would be needed to determine if their baseline serum TK1 levels could predict which individuals have an increased risk for disease progression. Although such studies have not been conducted, similar studies have indicated that tumor TK1 expression and tumor TK1 activity can predict which tumors have an increased risk for recurrence (20-22).

Overall, it is clear that this novel TK1 ELISA is both sensitive and specific for the early detection of lung cancer. Further studies are needed to validate these exciting preliminary results. We believe this ELISA will aid in the early detection of lung cancer and the early detection of recurrent lung tumors. TK1 activity as detected by the traditional radioassay has already proven efficacious in these areas. Therefore, we believe that this novel TK1 ELISA will similarly prove effective in monitoring a patient's response to treatment.

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