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Original Article

Elevated levels of CXC chemokine connective tissue activating peptide (CTAP)-III in lung cancer patients

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Abstract: Despite advances in treatments, lung cancer has been the leading cause of cancer-related deaths in the United States for the past several decades. Recent findings from the National Lung Screening Trial reveal that low-dose helical computed tomography (CT) scan screening of high-risk individuals reduces lung cancer mortality. This suggests that early detection is of key importance to improving patient outcome. However, of those screened with CT scans, 25% had positive scans that require further follow-up studies which often involve more radiation exposure and invasive tests to reduce false positive results. The purpose of this study was to identify candidate plasma biomarkers to aid in diagnosis of lung cancer in at-risk individuals. We found increased expression of the CXC chemokine connective tissue-activating peptide (CTAP)-III from plasma specimens of lung cancer patients compared to at-risk control subjects. Identification of the peptide was confirmed by the addition of an anti-NAP-2 antibody that recognizes CTAP-III and NAP-2. We also quantified and verified the increased levels of plasma CTAP-III with ELISA in patients with lung cancer (mean ± SD, 1859 ± 1219 ng/mL) compared to controls (698 ± 434 ng/mL; $P<0.001$). Our findings demonstrate elevated plasma levels of CTAP-III occur in lung cancer patients. Further studies are required to determine if this chemokine could be utilized in a blood-based biomarker panel for the diagnosis of lung cancer.

Keywords: Lung cancer, connective tissue activating peptide (CTAP)-III, neutrophil activating peptide (NAP)-2, CXC chemokines

Introduction

In 2010, over 220,000 new cases of lung cancer were diagnosed and approximately 150,000 lung cancer-related deaths were reported in the United States (US) [1]. Despite advances in treatments, lung cancer has been the leading cause of cancer-related deaths in the US with an unchanged overall survival of about 15% for the past several decades. More than 75% of patients with lung cancer will have locally advanced or metastatic disease at presentation with an associated 5 year survival of less than 5% [2]. It is well known that cigarette smoking accounts for 85-90% of lung cancer deaths [3]. There are approximately 90 million individuals in the US with a history of cigarette smoking and nearly half of these are current smokers. This indicates that lung cancer remains a major public health problem [2].

On the basis that clinical stage at diagnosis is a major determinant of survival after therapy and that treatment may be more effective if lung cancer could be detected at an earlier stage, the National Lung Screening Trial (NLST), randomized more than 53,000 current and former heavy smokers ages 55 to 74 years, compared the effects of low-dose helical computed tomography (CT) and standard chest X-ray on lung cancer mortality, and found 20% fewer lung cancer deaths in participants screened with low-

dose helical CT [4]. This study suggests that early detection is of key importance to improving patient outcome. However, of those screened with CT scans, 25% had positive scans that required follow-up studies. These additional studies often involved radiation exposure and invasive tests, including lung surgery.

A blood-based biomarker is an attractive, relatively non-invasive modality that could complement technologies, such as helical CT scans, in facilitating early detection and treatment of lung cancer. Biomarkers may help in the identification of individuals with radiographic abnormalities that should undergo further invasive testing. The purpose of this study is to identify candidate plasma biomarkers to aid in the diagnosis of lung cancer in at-risk individuals. Here, we report increased expression of the CXC chemokine connective tissue-activating peptide (CTAP)-III from plasma specimens of lung cancer patients compared to at-risk control subjects.

Materials and methods

Plasma biospecimens

The UCLA Lung Cancer Research Program has established a specimen bank with prospective collection of tumor, blood, and urine from subjects seen at UCLA and UCLA-affiliated hospitals and clinics. These specimens are complemented with a collection of general health and medical information, including clinical and pathologic stages, medication history and comorbidities. Control plasma specimens utilized in this study were collected from individuals at risk of developing lung cancer ($\geq$ 30 pack-years history of tobacco use, age $\geq$ 45 years, and smoking cessation of at least 1 year). Control subjects were screened for presence of lung cancer with a comprehensive work up that was comprised of blood work (complete blood count, chemistry panel, and coagulation studies), spirometry, helical CT scans and Lung Imaging Fluorescence Endoscope (LIFE) bronchoscopy with bronchoalveolar lavages and bronchial biopsies. Demographics, smoking history, environmental exposures, and quality of life instruments were collected and archived from all participants. These control subjects all underwent the most comprehensive diagnostic procedures available to rule out pre-existing lung cancer (combination of chest CT and fluorescence bronchoscopy) and consist of subjects at elevated risk for developing lung cancer due to smoking history and age. Thus, the specimens utilized in these studies accurately represent the clinical scenario that would benefit from a test for the early detection of lung cancer.

Identification of candidate protein biomarkers using Protein Chip immunoassays

The protein profiles between 30 non-small cell lung cancer (NSCLC) patients and 30 high-risk controls were obtained utilizing the Ciphergen Autobiomarker system (Freemont, CA, USA). For initial analyses plasma samples were applied to protein chips in a bioprocessor that contained chemically [anionic, cationic, immobilized metal affinity (IMAC)] modified surfaces. Subsequent profiling was performed on IMAC surfaces because these chips were the most discriminatory. For each sample 3 μL of plasma was applied to the protein chip in 100 μL of binding buffer. After a 60-minute incubation, the chips were washed to remove nonspecifically bound proteins, thus, allowing the capture of specific subsets of proteins from the sample. The proteins bound to the chip were then analyzed in a Ciphergen Protein Biomarker System (PBS) II. The resulting protein profiles were then analyzed with Ciphergen Biomarker Patterns software to identify discriminatory protein peaks.

In plasma protein profiles obtained from comparison of cancer and control samples, the abundance of a protein at mass-to-charge ratio (m/z) 9,280 was elevated in cancer samples compared to control samples. In order to identify this protein we utilized 1 mL of plasma collected from one of our cancer subjects. The plasma was denatured with 1 mL of urea buffer (9 M urea, 2% CHAPS, 50 mM Tris-HCl pH 9) for 30 min at 4°C. This sample was then diluted with 2.5 mL of 50 mM Tris-HCl pH 9.0 buffer. The denatured sample was then added to 2 mL of hyper Q beads (BioSepra) and allowed to bind for 30 min at room temp. The sample was then centrifuged and the supernatant collected. The beads were then resuspended in 6 mL of 50 mM Tris-HCl pH 9.0 buffer, mixed for 10 min at room temperature and centrifuged. This was repeated with buffers of pH 7, 5, 4, 3, and an organic wash (33% isopropanol, 17% acetonitrile, 0.1% triflouroacetic acid). These seven fractions were then spotted on IMAC and NP20 protein chips and read in a PBS IIc protein chip reader to identify fractions enriched for the 9,280 m/z peak. Enriched fractions were then applied to a column packed with IMAC30 beads

(BioSepra) 0.4 mL bed volume. The column was eluted with a batch-step gradient (3x0.4 mL of 5, 10, 20, 30, 40, and 50 mM imidizol in HEPES pH 7 buffer). The flow was stopped for 10 minutes at each elution step. The fractions were collected and again the isolation of the 9.28 kDa protein was assessed by mass spectroscopy with IMAC and NP20 chips. The fraction enriched for the 9.28 kDa protein was then applied to reverse phase beads (BioSepra) and eluted with a step gradient increasing from 10 to 60% acetonitrile 0.1% TFA. The fraction enriched for the 9.28 kDa protein was then separated by SDS-PAGE. The gel was stained with colloidal coomassie blue and the band corresponding to the 9.28 kDa protein was cut out. The gel band was passively eluted and a portion was applied to a NP20 protein chip to confirm that the isolated protein was the target protein. The protein was digested with trypsin and the digest was applied to a NP20 chip. The chip was analyzed in Q-TOF MS/MS equipped with a Ciphergen protein chip interface. The protein was identified as CTAP-III. This was confirmed by binding the anti-NAP2 antibody (rabbit anti-human NAP-2 polyclonal antibody, Chemicon, Temecula, CA) to a Ciphergen PS20 chip and pulling down the 9.28 kDa protein. In addition, an immunoprecipitation was performed to deplete the 9.28kDa protein from a cancer patient plasma sample.

Quantification of CTAP-III/NAP-2 levels by ELISA

We quantified the plasma levels of CTAP-III in 28 lung cancer patients and 28 at-risk controls using an ELISA kit against human CTAP-III/NAP-2 in accordance with the manufacturer's instructions (DuoSet, R&D Systems, Minneapolis, MN, USA).

Statistical analyses

The statistical handling of the SELDI involves a two-step process: data pre-processing and analysis. Data pre-processing for SELDI involves a series of steps: baseline subtraction, normalization, spectrum alignment, binning, and peak detection. We employed the Ciphergen SELDI software version 3.1 to perform data acquisition and preprocessing. Our analysis strategy for the SELDI data was to first identify those protein peaks that were differentially expressed in cancer versus non-cancer. For each marker we used the two sample t-test model to compare protein peaks between the groups. Next, the significant markers were combined together and forwards stepwise regression was used to construct a final model with multiple markers. A ROC curve was constructed based on the fitted values from the multiple logistic regression model and the area under the curve (AUC) was computed by numerical integration. For the ELISA data we compared CTAP-III/NAP-2 levels between cancer and control patients with the Wilcoxon rank sum test due to the non-normality of the expression values. A ROC curve was also constructed for the CTAP-III/NAP-2 expression values and the AUC was computed as above. Statistical analyses were performed using SAS and S+ statistical analysis software.

Results

Patient population characteristics

The protein profiles between 30 NSCLC patients and 30 high-risk controls were analyzed (**Table 1**). Plasma specimens utilized in this study were collected from lung cancer patients and control subjects who were at risk of developing lung cancer ($\geq$ 30 pack-years of tobacco use, age $\geq$ 45 years, and smoking cessation of at least 1 year).

Protein peak identification

Using the Ciphergen software, we identified 25 peaks that were differentially expressed ($P<0.05$) between lung cancer patients and controls. The first step of the analysis utilized a t-test to compare the expression at each peak between lung cancer and high-risk controls. Next, we used a stepwise model selection to identify a set of peaks that best predicted cancer status using the set of peaks identified by the t-test. The model used three markers (m/z 8,142, 9,287, and 79,584). Next, we constructed an ROC curve based on these multivariate models using the predicted probabilities of cancer and the area under the ROC curve was 0.93 (data not shown). These results indicate that we can very accurately predict cancer status on the basis of a small number of marker proteins.

Purification and identification of candidate protein biomarkers

In order to identify these proteins, we took

Table 1. Patient Population Characteristics

Demographic Variables	Controls (n=30)	Cancer (n=30)
Age, years (mean)	56	66
Gender		
Male	15 (50%)	19 (63%)
Female	15 (50%)	11 (37%
Tobacco use (former or current)	30 (100%)	23 (77%)
Adenocarcinoma	---	20
Squamous cell carcinoma	---	6
Mixed adenocarcinoma-squamous	---	1
NSCLC, NOS	---	3
Stage		
0	---	
I	---	7
II	---	2
III	---	5
IV	---	14
Unknown	---	2

* NOS, Not otherwise specified

advantage of the protein chip chemistries to isolate the protein of interest followed by the SDS-PAGE purification and final tandem MS/MS identification on a Sciex Q-TOF instrument. In plasma protein profiles obtained from cancer and control samples as described above, the abundance of a protein with a approximate m/z ratio of 9,280 was elevated in cancer samples compared to control samples. We have confirmed the identity of this protein by binding the anti-NAP2 antibody to a Ciphergen PS20 chip and pulling down the 9.28 kDa protein. In addition, an immunoprecipitation was performed to deplete the 9.28 kDa protein from a cancer patient plasma sample (**Figure 1**).

Measurement of CTAP-III protein using ELISA

The plasma levels of CTAP-III were quantified in 28 patients with lung cancer and in 28 at-risk control subjects using ELISA (**Figure 2**). CTAP-III was found to be elevated in patients with lung cancer (mean ± SD, 1859 ± 1219 ng/mL) in comparison with control subjects (698 ± 434; $P<0.001$). The receiver operating characteristic curve for CTAP-III/NAP-2 is shown in **Figure 3** and the area under the curve is 0.83.

Discussion

The recent findings from the NLST revealed that low-dose helical CT scan screening of high-risk individuals reduced lung cancer mortality. However, there are potential disadvantages of helical CT scans including 1) the cumulative exposure to radiation from multiple CT scans, 2) risks and potential complications associated with invasive testing (i.e. needle biopsy or lung surgery) for evaluation of patients who prove not to have lung cancer, and 3) significant patient anxiety and expense to healthcare generated from suspicious findings that ultimately reveal no cancer in most cases [2, 4]. Therefore, a non-invasive modality that is cost-effective and accurate in predicting lung cancer is needed to complement technologies, such as helical CT, in lung cancer screening, risk stratification, and monitoring for recurrent disease. To date, there has not been a validated biomarker shown to have adequate sensitivity, specificity, and reproducibility to be used as a biomarker for the early detection and treatment of lung cancer. In this report, we have identified the peptide CTAP-III was significantly elevated in lung cancer patients, and confirmed its identification with depletion of the peptide by the addition of anti-NAP-2 antibody which recognizes CTAP-III and NAP-2. We also quantified and verified the increased levels of plasma CTAP-III with ELISA in patients with lung cancer (mean ± SD, 1859 ± 1219 ng/mL) compared to control subjects (698 ± 434 ng/mL; Wilcoxon rank sum test $P<0.001$; Area under the ROC curve = 0.83).

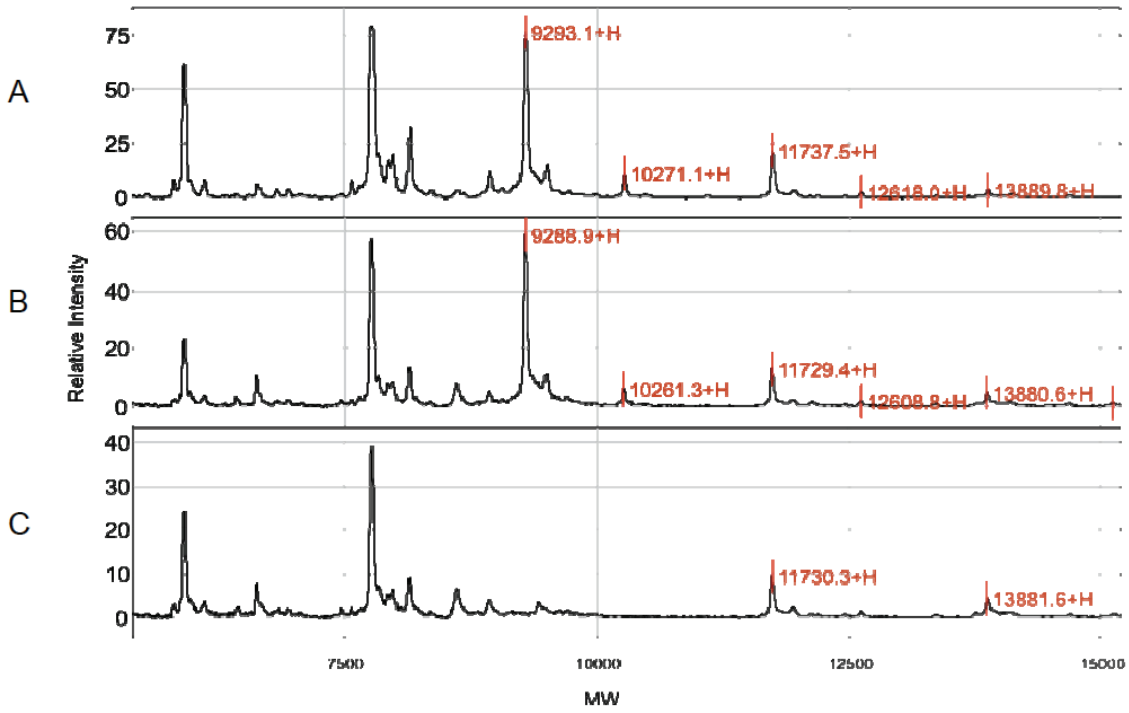


Figure 1. Isolation of elevated protein with a mass-to-charge (m/z) ratio of ~9,280 in a patient with lung cancer. Antibody to NAP-2 leads to loss of peak (9,280) by mass spectroscopy. *A.* No antibody *B.* Rabbit IgG control antibody *C.* Anti-NAP-2 antibody.

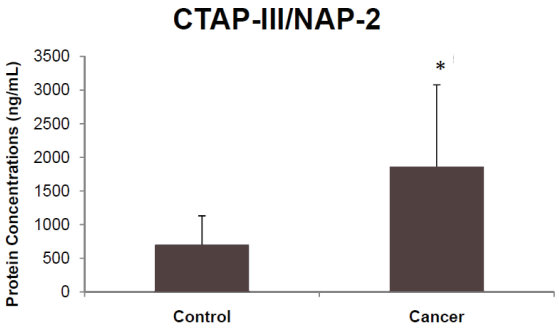


Figure 2. Serum CTAP-III/NAP-2 levels as determined by ELISA. Serum CTAP-III/NAP-2 levels were significantly elevated in lung cancer patients compared to high-risk controls. (*Wilcoxon rank sum test *P value* < 0.001; Error bars are ± 1 S.D.)

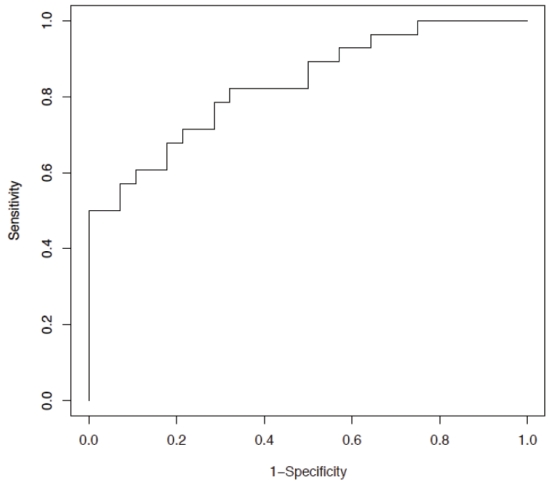


Figure 3. The receiver operating characteristics for CTAP-III/NAP-2 in individuals with lung cancer versus high-risk controls. Area under the curve = 0.83.

CTAP-III is a peptide made up of 85 amino acids with a molecular weight of 9,278 Daltons and is secreted from activated platelets, macrophages, neutrophils and lymphocytes [5, 6]. Transcription of the leukocyte-derived growth factor gene gives rise to platelet factor 4 (PF4) and polypeptide beta-thromboglobulin antigen (β-thromboglobulin Ag), which can be proteolytically cleaved to form the following peptides: platelet basic protein (PBP), CTAP-III, and β-thromboglobulin [7-9]. While CTAPs were originally named for their ability to synthesize ex-

tracellular matrix glycosaminoglycans in connective tissue cells, CTAP-III plays a role in recruitment of neutrophils [9, 10], antimicrobial activities [11, 12], histamine release [13] and glucose transport [14].

Interestingly, CTAP-III is readily cleaved by the proteases produced by neutrophils and mast cells to form NAP-2, a potent chemokine for neutrophil chemotaxis, neutrophil-endothelial cell adhesion, transendothelial migration, and inhibit megakaryocytopoiesis in a negative feedback mechanism [8, 9, 15]. NAP-2, also known as CXCL7, has a higher affinity to bind to chemokine receptor CXCR2 than to CXCR1 depending on levels of NAP-2 [8, 16]. These receptors also bind to other CXC chemokines such as interleukin 8 (IL8) and growth regulated gene (Gro) [17]. Crosstalk between platelet-derived chemokines modulates the effects of the CXC chemokines. For example, downregulation of receptor CXCR2 occurs on neutrophils in the presence of CTAP-III, resulting in reduced CXCL7 -mediated effects [8].

The 94-residue precursor polypeptide platelet basic protein (PBP) gives rise to three smaller peptides, also known collectively as the beta-thromboglobulin-antigen (b-thromboglobulin-Ag), and is comprised of CTAP-III, NAP-2 (also known as CXCL7), and β-thromboglobulin [7]. While CTAP-III is the predominant β-thromboglobulin-Ag component stored in platelet granules, NAP-2 is readily formed (within minutes) from the cleavage of CTAP-III by neutrophil enzyme cathepsin G or mast cell chymase [5, 18]. Both CTAP-III and NAP-2 appear early in inflammatory states to recruit neutrophils and promote transendothelial migration as well as inhibit megakaryocytopoiesis. Although primarily present in platelets, activated macrophages, neutrophils, and lymphocytes, CXLC7 was found to be one of several factors found in the secretome of early pro-angiogenic cells or endothelial progenitor cells [19].

CTAP-III and NAP-2 from platelets have previously been reported to demonstrate heparanase activity and degrade heparan sulfates [20], which are major components of basement membranes and cell surfaces, but the specific consequences in cancer pathology are still not well elucidated. CXCL7 expression was found to be increased in malignant breast cancer cells and conferred invasive properties in CXCL7-transfected breast cells [21]. These transfected cells demonstrated elevated heparanase enzymatic activity which could alter the extracellular matrix and thus facilitate cancer metastasis.

CXCL7 and its receptor CXCR2 contribute important roles in tumor growth and development as well as tumor angiogenesis in lung cancer and other cancer cell types [22-24]. Co-culture of bone marrow-derived mesenchymal cells and breast cancer stem cells induced mRNA expression of several cytokines including CXCL7, CXCL6 (GCP2), CXCL5 (ENA78), CXCL1 (Gro-a), IL6 and IL8, many of which are ligands to the CXCR1 and CXCR2 receptors [25]. Liu et al showed that only anti-CXCL7 antibody reduced the breast cancer stem cell population in co-culture conditions, inhibited mesenchymal stem cell chemotaxis, and that CXCL7 production by mesenchymal stem cells was induced by IL6. CXCR2 serves as a receptor for several angiogenic ELR+ CXC chemokines described earlier including CTAP-III and CXCL7. Depletion of CXCR2 in lung cancer animal models inhibited tumor growth, reduced vascular density and induced tumor necrosis [22].

CTAP-III/NAP-2 has been studied in other diseased states as a potential biomarker. Yee et al. reported increased CTAP-III/NAP-2 levels from lung cancer patients prior to surgical resection and levels decreased significantly after surgery [26]. They also showed that elevated CTAP-III/NAP-2 levels were present in plasma collected before clinical presentation of lung cancer. The subjects were part of the Lung Health Study, a lung cancer screening prevention study. Another study examined six inflammatory markers in patients with chronic obstructive pulmonary disease (COPD), which is a chronic inflammatory disease of the airways and the lung parenchyma [27]. They reported no difference in NAP-2 levels of individuals with or without COPD. These findings, along with results from Yee et al [26], suggest that elevated CTAP-III/NAP-2 levels may predict lung cancer in at-risk individuals who have COPD, a known risk factor for lung cancer [28-32].

In summary, we report increased expression of the CXC chemokine CTAP-III from plasma specimens of lung cancer patients compared to at-risk control subjects, suggesting that this chemokine could potentially be a blood-based biomarker associated with lung cancer. Future

studies are warranted to validate CTAP-III as a sensitive and specific diagnostic marker for discriminating individuals with lung cancer from other cancers or inflammatory conditions. We are currently exploring additional plasma biomarkers to be used in conjunction with CTAP-III to aid in early lung cancer detection.

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