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**Tumstatin, a Matrikine Derived from Collagen Type IVα3, is Elevated in Serum from Patients with Non–Small Cell Lung Cancer**

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**Abstract**

**OBJECTIVES:** Fibrosis and cancer are characterized by extracellular matrix (ECM) remodeling. The basement membrane is mainly composed by collagen type IV and laminin. Tumstatin is a matrix metalloproteinase-9 (MMP-9) generated matrikine of collagen type IV α3 chain. We evaluated the potential of tumstatin as a diagnostic biomarker of lung disorders.

**METHODS:** A monoclonal antibody was raised against the neo-epitope tumstatin. A novel competitive enzyme-linked immunosorbent assay for detection of tumstatin (TUM), was developed and technically characterized. Levels of TUM were measured in serum of patients with idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), and non–small cell lung cancer (NSCLC) belonging to two cohorts.

**RESULTS:** The developed TUM enzyme-linked immunosorbent assay (ELISA) was technically robust. In cohort 1, levels of TUM were significantly higher in NSCLC compared to healthy controls, IPF, and COPD (P = 0.007, P = 0.03 and P = 0.001, respectively). The area under the receiver operating characteristics (AUROC) for separation of patients with NSCLC from healthy controls was 0.97, for separation of NSCLC and IPF patients was 0.98, and for separation of NSCLC and COPD patients was 1.0. In cohort 2, levels of TUM were also significantly higher in patients with NSCLC compared to healthy controls (P = 0.002), and the AUROC for separation of NSCLC and healthy controls was 0.73.

**CONCLUSIONS:** We developed a technically robust competitive ELISA targeting the fragment tumstatin. The level of TUM in circulation was significantly higher in patients with NSCLC compared to patients with IPF, COPD and healthy controls. The assay provided high diagnostic accuracy in separating NSCLC patients from other lung disorders and from healthy controls.

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**Introduction**

The extracellular cellular matrix (ECM) is the noncellular component of all organs and tissues. Altered ECM remodeling plays a vital role in diseases such as fibrosis and cancer [1]. The basement membrane (BM) is a specialized ECM which functions as a scaffold for epithelial and endothelial cells, acts as a barrier between tissues, and supports tissue structure by maintaining epithelial and endothelial cell polarity and function [2,3]. The BM also serves as a barrier for cancer cell invasion. Breaching of the BM and loss of BM integrity are associated with an invasive cancer phenotype [4]. The BM is also remodeled as part of angiogenesis. During angiogenesis, the endothelial cells are induced to proliferate and migrate by signaling molecules generated by the degradation of the adjacent BM by proteolytic enzymes. BM degradation leads in fact to exposure of protein domains (so called “cryptic sites”) or protein fragments with either proangiogenic or antiangiogenic activity. One of the best-described fragments is the matrikine endostatin, a known inhibitor of angiogenesis and tumor growth [5].

The two main BM proteins are collagen type IV and laminin, which together form a distinct network linked together by nidogen and heparin sulfates [3,6–8]. Collagen type IV is formed by the...
combination in heterotrimeric of six different α-chains (α1–6) in the mammalian BMs [9]. The α3 chain of collagen type IV (COL4α3) has been described to have restricted distribution across BMs and is primarily found in the lungs and kidneys [10]. The structural role of COL4α3 is illustrated by clinical manifestations of Alport’s syndrome, Goodpasture’s syndrome, idiopathic pulmonary fibrosis (IPF), and lung cancer, which are characterized by damage to COL4α3 by either mutations, immune attacks or leaky vasculature and altered BM composition [11–14].

Tumstatin is a 28-kDa fragment of COL4α3 that binds to endothelial cells via the αvβ3 integrin [15]. It is a matrikine generated by matrix metalloproteinase-9 (MMP-9) and known to keep endothelial cells via the intravenously with 50 μl of 0.9% NaCl. MMP-9 is needed to cleave tumstatin from α3 collagen and function as a protective matrikine. Lack of MMP-9 accelerates tumor growth in MMP-9 knockout mice, while high COL4α3 expression correlates with poor prognosis in patients with lung cancer [17,18]. This collagen chain therefore has both protective and tumor-promoting capabilities.

Several studies have hypothesized the potential of matrikines, such as tumstatin, as biomarkers of pathology; however, no such marker is currently available [13,19–21]. Our hypothesis was that tumstatin is a potential biomarker for lung disorders reflecting degradation of the BM. We therefore developed and validated a novel neo-epitope specific competitive enzyme-linked immunosorbent assay (ELISA) targeting tumstatin (TUM), and measured the concentration of TUM in serum samples of patients diagnosed with different lung disorders, including IPF, chronic obstructive pulmonary disease (COPD), and non–small cell lung cancer (NSCLC) and investigated its potential as a diagnostic biomarker.

Materials and Methods
All reagents used for the experiments were high-quality standards from Sigma Aldrich (St. Louis, MO) and Merck (Whitehouse Station, NJ). The synthetic peptides used for immunization and assay development were purchased from American Peptide Company (Sunnyvale, CA).

Generation of Monoclonal Antibodies
The amino acid sequence PGLKGKRGDS in the human α3 chain of type IV collagen was used for the generation of monoclonal antibodies (mAbs). The corresponding sequence in rat has one mismatch in position 6, and in mouse, it has a mismatch in position 5 (Figure 1). Immunization was initiated by subcutaneous injection of 200 μl emulsified antigen and rested for a month. Subsequently, the mouse was boosted by serial dilution of the standard peptide and plotted using a four-parametric mathematical fit model. Standard concentrations were 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 ng/ml. Each plate included five kit controls to monitor intra- and interassay variation. All samples were measured within the range of the assay, and all samples below lower limit of measurement range (LLMR) were reported as the value of LLMR.

** Figure 1. Sequence alignment between human, mouse, and rat tumstatin. The antibody recognizes residues from 1426 to 1436.**

**TUM ELISA**
The TUM competitive ELISA procedure was as follows: 96-well streptavidin-coated ELISA plates (Roche, cat. 11940279) were coated with 10 ng/ml biotinylated peptide PGLKGKRGDS-K-Biotin dissolved in assay buffer (25 mM Tris-BTB 2g, NaCl/L, pH 8.0), and incubated for 30 minutes at 20°C in the dark with 300 rpm shaking. Plates were washed five times in washing buffer (20 mM TRIS, 50 mM NaCl, pH 7.2). Subsequently, 20 μl of standard peptide or sample was added to appropriate wells, followed by 100 μl of 7 ng/ml horseradish peroxidase–labeled monoclonal antibody solution. The plates were incubated for 1 hour at 20°C with 300 rpm shaking and subsequently washed in washing buffer. Finally, 100 μl 3,3',5,5-tetramethylbenzidine (TMB) (Kem-En–Tec cat. 4380H) was added and incubated for 15 minutes at 20°C with 300 rpm shaking. To stop the enzyme reaction of TMB, 100 μl of stopping solution (1% H2SO4) was added. The plate was analyzed by an ELISA reader at 450 nm with 650 nm as reference (VersaMax; Molecular Devices, Sunnyvale, CA). A standard curve was performed by serial dilution of the standard peptide and plotted using a four-parametric mathematical fit model. Standard concentrations were 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 ng/ml. Each plate included five kit controls to monitor intra- and interassay variation. All samples were measured within the range of the assay, and all samples below lower limit of measurement range (LLMR) were reported as the value of LLMR.
Technical Evaluation

Two-fold dilutions of four human serum and human urine samples were used to assess linearity. Linearity was calculated as a percentage of recovery of the undiluted sample.

Antibody specificity was calculated as a percentage of signal inhibition by two-fold diluted standard peptide (PGLKGKRGDS), elongated peptide (LPGLKGKRGDS), truncated peptide (GLKGKRGDS), and non-sense peptide (LRISKKKFRR). The intra- and interassay variation was determined by 10 independent runs of five quality controls and two kit controls run in double determinations. Accuracy of the assay was measured in healthy human serum/urine samples spiked with standard peptide and a serum/urine sample with a known high TUM concentration, and calculated as the percentage recovery of the measured value and the expected concentration of the peptide or the serum/urine sample with high TUM plus the concentration of the analyte in serum/urine. Interference was measured in healthy human serum spiked with biotin (low = 30 ng/ml, high = 90 ng/ml), hemoglobin (low = 0.155 mM, high = 0.310 mM), or lipids (low = 4.83 mM, high = 10.98 mM). The interference was calculated as the percentage recovery of the analyte in nonspiked serum. Furthermore, the interfering effect of human anti-mouse antibody (HAMA) was evaluated. Five healthy human serum samples were added to a panel of different HAMA concentrations. These were analyzed with and without 5% Liquid II (Osteocalcin ELA Puf-Liq by Roche Diagnostics) in the dilution buffer, which counteracts the interference by HAMA. Salt interference was tested by measuring salt samples with a concentration of 8.14 g/l NaCl at pH 7.0 and 8.0. To define the concentration of TUM in the healthy population, 32 healthy human serum samples were measured, and the relation of the marker concentration to age and gender of the sample donors was evaluated. LLMR and upper limit of measurement range (ULMR) were calculated based on the 10 individual standard curves from the intra- and interassay variation. The analyte stability was determined for three healthy human serum samples incubated at either 4 or 20°C for 2, 4, and 24 hours. The stability of the samples was evaluated by calculating the percentage variation from the sample kept at 20°C (0 hour sample). Furthermore, the analyte stability was determined for three healthy human serum samples exposed to four freeze and thaw cycles. To assess the stability of the analyte, the percentage recovery of the analyte was calculated from the sample that underwent only one freeze/thaw cycle.

Biological Validation of TUM

TUM was measured in serum samples from two different cohorts. Both cohorts were obtained from the commercial vendor Proteogenex (Culver City, CA). Samples were collected after informed consent and approval by the local Ethics Committee and in compliance with the Helsinki Declaration of 1975.

Cohort 1 included patients diagnosed with IPF, COPD, NSCLC and colonoscopy-negative controls with no symptomatic or chronic disease. Patient demographics are shown in Table 2. Cohort 2 included patients diagnosed with NSCLC in cancer stage I, II, III, and IV together with colonoscopy-negative controls with no symptomatic or chronic disease. Patient demographics of this cohort can be found in Table 3.

Ethical Statement

The production of monoclonal antibodies performed in mice was approved by the National Authority (The Animal Experiments Inspectorate) under approval number 2013-15-2934-00956. All animals were treated according to the guidelines for animal welfare.

Statistical Analysis

Characteristics of the cohorts are presented as a number (frequency) and percentage for categorical variables and mean (standard deviation) for continuous variables. Statistical differences for categorical were assessed using a Kruskal-Wallis test (nonparametric) for cohort 1 and a Mann-Whitney t test in cohort 2. Results are shown as a Tukey boxplots. The diagnostic power of TUM was investigated by the area under the receiver operating characteristics (AUROC) curve. For all statistical analyses performed, a P value below .05 was considered significant. Asterisks indicate the following: *P < .05; **P < 0.01. Statistical analysis and graphs were performed using GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA).

Results

Clone Characterization

The best antibody producing hybridomas were screened for reactivity towards the standard peptide and native material in the competitive ELISA. Based on the reactivity, the clone NBH134#102-3GF was chosen for assay developed and determined to be the IgG1 subtype. Native reactivity was observed in human serum and urine (Figure 2), while no reactivity was found towards the elongated peptide, truncated peptide, non-sense standard peptide, and non-sense coater (Figure 3).

Technical Evaluation of the TUM ELISA Assay

A full technical validation was performed to evaluate the TUM ELISA assay. A summary of the validation data can be found in Table 1. The measurement range (LLMR-ULMR) of the assay was determined to be 0.26 to 9.92 ng/ml. The intra- and intervariation was 8.04% and 10.96%, respectively, based on 10 independent assays. Linearity of the human samples was observed from undiluted to four-fold dilution for human serum, and undiluted to two-fold dilution for human urine. Spiking recovery of standard peptide in human serum, and human serum in human serum resulted in a mean recovery of 90% and 99%, respectively. Hemoglobin, lipids, and biotin did not interfere with measurements of the TUM analyte in human serum. The stability of...
the analyte was acceptable during both prolonged storage of human serum samples at 4°C and 20°C (102.4% and 80.1%) and during freeze/thaw cycles (80.8%).

**Biological Evaluation – TUM as a Biomarker for Lung Cancer**

TUM was measured in serum of patients with lung disorders from two independent cohorts: cohort 1 and cohort 2.

Cohort 1 consists of healthy controls and patients diagnosed with IPF, COPD, and NSCLC. TUM was significantly elevated in serum from NSCLC compared to healthy controls, IPF patients, and COPD patients (P = 0.007, P = 0.03, and P = 0.001, respectively) (Figure 4). No significant difference was observed between healthy controls, IPF patients, and COPD patients, which indicated that TUM may play a role in NSCLC but not in fibrotic lung disorders.

In cohort 2, TUM was measured in samples from healthy controls and patients with NSCLC. TUM was significantly upregulated in patients with NSCLC compared to healthy controls (P = .002) (Figure 5). TUM was not significantly different between the different cancer stages.

The AUROC was used to evaluate the diagnostic power of TUM to separate NSCLC and healthy controls. As shown in Table 4, TUM was able to discriminate between NSCLC patients and healthy controls in cohort 1 with an AUROC of 0.97, NSCLC patients and IPF patients with an AUROC of 0.98, and NSCLC and COPD patients with an AUROC of 1.00.

In cohort 2, TUM was able to discriminate between NSCLC patients and healthy controls with an AUROC of 0.73. We furthermore saw that diagnostic accuracy increased with disease stage. These findings indicate that serological TUM levels could be used to separate healthy controls from patients with NSCLC with a high diagnostic accuracy.

**Discussion**

In the present study, we developed and characterized a novel competitive ELISA for the detection of tumstatin (TUM) using a monoclonal antibody detecting a MMP-9 generated neo-epitope fragment of the alpha 3-chain of collagen type IV corresponding to the bioactive molecule tumstatin. The main findings of this study were: i) the assay was technically robust and specific towards the human tumstatin sequence PGLKGKRGDS, ii) the fragment was detectable in human serum and urine and iii) the fragment was significantly elevated in patients with NSCLC compared to IPF patients, COPD patients, and healthy controls, with a high diagnostic accuracy. To our knowledge, this is the first study to develop and validate a specific biomarker of tumstatin and validate it in patients with lung disorders.

TUM was characterized as being a technically robust and sensitive assay, with an IC50 value of 1.6 ng/ml and measurement range from 0.26 ng/ml to 9.92 ng/ml. All technical tests including intra- and intervalation, dilution recovery, interference, and analyte stability gave results within the acceptable range, and the assay specificity test showed that the monoclonal antibody was specific towards the

| Table 1. TUM ELISA Technical Validation Data |
|---------------------------------------------|
| Technical Validation Test                  | Result                       |
| IC50                                        | 1.6 ng/ml                    |
| Detection range                            | 0.26–9.92 ng/ml              |
| Intraassay variation                        | 8.04%                        |
| Interassay variation                       | 10.96%                       |
| Dilution recovery of human serum            | 89%                          |
| Dilution recovery of human urine            | 98%                          |
| Analyte recovery 24 h, 4°C/20°C             | 102.4%/80.1%                 |
| Hemoglobin recovery, low/high               | 100%/100%                    |
| Lipemia recovery, low/high                  | 100%/100%                    |
| Biotin recovery, low/high                   | 120%/106%                    |
| Salt recovery, pH 6.0/pH 7.0/pH 8.0        | 97%                          |
| Spiking recovery (peptide in serum)         | 90%                          |
| Spiking recovery (serum in serum)           | 99%                          |
| Analyte recovery, 4 freeze/thaw cycles      | 80.8%                        |

* Percentages are reported as mean.  
* Average recovery after salt interference.

| Table 2. Patient Demographics of Cohort 1 |
|------------------------------------------|
| Healthy Controls (n = 8)                 | IPF (n = 7)                  | COPD (n = 8)    | NSCLC (n = 8)  | P Value     |
| Age                                      | 54.88 (7.85)                 | 74.13 (8.36)    | 75.38 (1.69)  | 60.50 (9.32) | <0.001  |
| Male, n (%)                              | 6 (75%)                      | 4 (57%)         | 4 (50%)       | 7 (87.5%)    | 0.102   |
| BMI                                       | 26.25 (1.27)                 | 25.79 (1.58)    | 27.24 (1.84)  | N/A          | 0.170   |
| FEV1% of predicted value                 | -                            | 64.38 (3.42)    | 61.50 (7.19)  | -            | 0.634   |
| FEV/FVC ratio %                          | 76.00 (1.51)                 | 58.38 (15.20)   | -             | 0.016        |
| TUM (ng/ml)                              | 1.79                         | 2.00            | 1.58          | 4.24         | <0.001  |

Data are presented as mean (SD) unless otherwise stated. Comparison of age, gender, BMI, and TUM levels was performed using Kruskal-Wallis adjusted for Dunn’s multiple comparisons test, while comparison of FEV1% of predicted value and FEV/FVC ratio % was calculated using the Mann-Whitney unpaired t test. P values below .05 were considered significant. Abbreviations: BMI, body mass index; FEV1, forced expiratory volume in 1 second; NSCLC, non–small cell lung cancer; FVC, forced vital capacity.

| Table 3. Patient Demographics of Cohort 2 |
|------------------------------------------|
| Healthy Controls (n = 20)                 | NSCLC (n = 40)               | P Value     |
| Age                                      | 61.85 (1.95)                 | 61.93 (2.14)  | 0.593   |
| Male, n (%)                              | 10 (50%)                     | 20 (50%)      | 1.000   |
| BMI                                       | 26.14 (2.67)                 | 25.55 (4.23)  | 0.533   |
| TUM (ng/ml)                              | 1.26                         | 2.00          | 0.002   |

Data are presented as mean (SD) unless otherwise stated. Comparison of age, gender, BMI, and TUM levels was performed using a Mann-Whitney t test. P values below .05 were considered significant.
cleavage site between amino acid 1425 and 1426 located in the alpha 3-chain of collagen type IV.

Our hypothesis was that tumstatin reflected the dysregulated turnover of the BMs [17]. Tumstatin was originally identified by Maeshima et al. [23] and named for its unique property of causing "tumor stasis." In that study tumstatin was shown to inhibit proliferation of vascular endothelial cells and tube formation by \( \text{in vitro} \) and \( \text{in vivo} \) models of angiogenesis and tumor growth [23], having therefore a protective role in cancer development. Decreased serum levels of Col. IV \( \alpha3 \) have previously been detected in MMP-9–deficient mice compared to normal mice [13]. This suggested that circulating tumstatin was only present as a consequence of MMP-9–mediated cleavage of COL4\( \alpha3 \). Both MMP-9 and COL4\( \alpha3 \) need to be upregulated for tumstatin to be generated and detected in human circulation. MMP-9 is known to be upregulated in cancer [24,25], and this could explain why levels of TUM are elevated in serum of patients with NSCLC. Since the fragment detected by TUM can only be found in serum when MMP-9 generates it, our results may suggest that NSCLC patients have a high MMP-9 activity, which increases the expression of tumstatin [13,18]. In line with this, MMP-9–generated type I collagen fragments have previously shown potential as biomarkers for NSCLC [26].

In this study, we showed that TUM has a diagnostic potential for NSCLC and is able to separate these patients from patients with lung fibrosis. Based on the high diagnostic accuracy, this could be a biomarker of BM remodeling in lung cancer. High levels of TUM in circulation may indicate disruption of the BM, which can favor cancer invasion [27]. Therefore, TUM may also serve as a marker of tumor activity and severity. As tumstatin appeared to have an antiangiogenic role, TUM may be used as a biomarker to assess the efficacy of antiangiogenic therapies. Antiangiogenic therapies have been extensively studied for the treatment of different cancer types including lung cancer. Despite many of them having been approved for the treatment of cancers, the overall survival benefit has been modest [28]. Biomarkers such as TUM may have the potential to identify the patients most or least likely to benefit from these drugs - a current medical need as there are no validated biomarkers for this purpose [29]. TUM may also be applied to study the intrinsic and acquired resistance mechanisms associated with these antiangiogenic compounds. Thus, based on the present findings and previously described role that many ECM/BM components and specific degradation fragments have in association to the pro- or antiangiogenic activity [17,27,30–38], TUM may have potential as a clinically relevant biomarker for predicting response to treatment. It could furthermore be used to study possible resistance mechanisms to anti-angiogenic compounds.

A limitation of the study lies in the relatively small population sizes and in the cross-sectional design. Moreover, we had limited clinical information on the patients. Nevertheless, we were able to confirm the findings from cohort 1 in cohort 2. Larger longitudinal studies are needed to fully evaluate the potential of TUM as a diagnostic and/or prognostic biomarker for NSCLC.

The assay was evaluated to be technical robust and not interfere with potential substances present in blood. However, whether the age of the samples or hemodialysis interferes with the assay was not analyzed, which is a limitation.
In conclusion, we developed the technically robust competitive ELISA TUM targeting a MMP-9-generated collagen type IV alpha-3 chain fragment known as tumstatin. TUM levels were significantly higher in serum of patients with NSCLC compared to patients with IPF, patients with COPD, and healthy controls. The assay provided high diagnostic accuracy in separating NSCLC patients from those with other lung disorders and from healthy controls. Since tumstatin acts as an inhibitor of tumor growth and has an antiangiogenic role, the marker may be used as a marker of angiogenesis and tumor formation.

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Conflict of interest
N. Willumsen, S. Sun, T. Manon-Jensen, M. A. Karsdal, and F. Genovese are employed at Nordic Bioscience A/S, which is a company involved in discovery and development of biochemical biomarkers. M. A. Karsdal owns stocks at Nordic Bioscience.

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